

Myogenic MicroRNA Expression Requires ATP-Dependent Chromatin Remodeling Enzyme Function^{▽†}

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Knockdown of the Brg1 ATPase subunit of SWI/SNF chromatin remodeling enzymes in developing zebrafish caused stunted tail formation and altered sarcomeric actin organization, which phenocopies the loss of the microRNA processing enzyme Dicer, or the knockdown of myogenic microRNAs. Furthermore, myogenic microRNA expression and differentiation was blocked in Brg1 conditional myoblasts differentiated ex vivo. The binding of Brg1 upstream of myogenic microRNA sequences correlated with MyoD binding and accessible chromatin structure in satellite cells and myofibers, and it was required for chromatin accessibility and microRNA expression in a tissue culture model for myogenesis. The results implicate ATP-dependent chromatin remodelers in myogenic microRNA gene regulation.

MicroRNAs (miRNAs) regulate the stability and processing of mRNAs and are significant posttranscriptional modulators of gene expression (2). miRNA function is involved in nearly all cellular processes examined, including development and tissue differentiation (24, 26). Components of the miRNA biogenesis pathway are similarly implicated in organismal development; the conditional ablation of the miRNA processing enzyme Dicer interferes with the differentiation and formation of numerous vertebrate tissue types (1, 4, 7, 8, 16, 19, 21, 22, 32, 33, 46). While there has been significant emphasis placed on characterizing miRNA formation and function, there has been considerably less examination of how the expression of miRNAs is regulated.

The family of mammalian SWI/SNF chromatin remodeling enzymes utilizes ATP hydrolysis to break histone-DNA contacts and alter genomic chromatin structure to regulate gene expression (15, 38, 44). SWI/SNF enzymes are involved in the differentiation of most tissue types, and they therefore are general regulators of tissue-specific chromatin accessibility and gene expression (13, 23). The role of the Brg1 ATPase and SWI/SNF enzymes in skeletal muscle differentiation has been investigated in depth, providing links between ATP-dependent chromatin remodeling enzymes, myogenic transcription factors, histone modification enzymes, and signal transduction pathways in the temporal control of myogenic gene activation (13, 18). However, because Brg1 is essential for mouse early development (5), mouse studies examining its specific function during skeletal muscle development have not been reported.

Genetic screens and the injection of morpholino oligonucle-

otides (MO) against specific genes into zebrafish early embryos provide an additional approach to examining function during development. The zebrafish *young* mutant, which has a defect in retinal differentiation, was mapped to the *brg1* locus, and targeting Brg1 by morpholino injection recapitulated the phenotype (17). Subsequent studies identified Brg1-dependent genes expressed during zebrafish retinal differentiation (27) and defined roles for Brg1 during zebrafish neural crest induction and neurogenesis (14). A recent study indicated that Brg1 was required for larval fin fold regeneration (47). However, there was no indication from these reports that the loss of Brg1 affected skeletal muscle formation, which seemed inconsistent with the literature based on primary and cultured myoblasts and skeletal muscle cells.

Here, we reexamined the phenotype of zebrafish embryos injected with Brg1 morpholinos. A significant subset of embryos presented with a short, stubby tail that showed altered somite structure and a disorganized muscle fiber structure. Remarkably, this skeletal muscle deficiency phenocopied the altered sarcomeric actin organization recently reported by Giraldez and colleagues when the miRNA processing enzyme, Dicer, was mutated and when specific myogenic miRNAs were targeted for knockdown (31). Thus, our results link the chromatin remodeling enzyme and miRNA function during skeletal muscle development. Our subsequent studies to probe the mechanism demonstrated that Brg1 and the MyoD master regulator of myogenesis were bound to the same regions upstream of miRNA stem-loop sequences and that functional Brg1 was required for chromatin accessibility at these sites and for miRNA expression. Our results indicate that one role for Brg1 in skeletal muscle development is to ensure the appropriate regulation of myogenic microRNA expression.

MATERIALS AND METHODS

RNA isolation and RT-PCR. RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA (0.5 µg) was used for reverse transcription (RT) reactions to generate cDNA using Moloney murine

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leukemia virus or superscript III (Invitrogen) reverse transcriptase enzymes as previously described (37). Quantitative PCR (qPCR) was performed with Qiagen hot start *Taq* master mix according to the manufacturer's protocol using MyoD and myogenin primers described previously (43). Primers for Ckm and primary microRNA transcripts are listed in Table S1 in the supplemental material. Amplification and quantification were performed using an MJ Opticon system.

Northern and Western blots. Total RNA was isolated from tissue or B22 cells at the indicated time points using TRIzol (Invitrogen), separated on a 15% acrylamide-urea gel, and transferred onto Hybond-XL membranes (Amersham Biosciences). The 5'- γ -³²P-end-labeled miR-1, miR-133a, or miR-29a oligonucleotide probes were hybridized to the membranes using Perfect Hyb plus hybridization buffer (Sigma). The blots were reprobed with labeled U6 snRNA as a loading control.

Western blots to detect FLAG-tagged dominant-negative Brg1 (DN Brg1) were performed on whole-cell extracts using rabbit polyclonal antisera against the FLAG epitope (12) exactly as described previously (10). The same procedures were used to measure Brg1 and phosphatidylinositol 3 (PI3) kinase levels using rabbit antisera against Brg1 (10) and commercial antibodies against PI3 kinase p85 (06-496; Millipore).

ChIP and restriction enzyme accessibility assay (REAA). Chromatin immunoprecipitations (ChIPs) were performed as previously described (9). The analysis of immunoprecipitated DNA was performed by quantitative real-time PCR using a Quantitect SYBR green PCR kit (Qiagen) and an MJ Opticon system. Oligonucleotides used for ChIP analyses are listed in Table S1 in the supplemental material. The antibodies used for the immunoprecipitation step included rabbit polyclonal antisera against Brg1 (10) and a commercial rabbit polyclonal antibody against MyoD (sc-304; Santa Cruz). IgG (Millipore) was used as a control. As an additional negative control, every sample was analyzed for the presence of the immunoglobulin H enhancer sequences; the detection of IgH sequences never exceeded background levels (data not shown).

REAs were performed as described previously (12, 34) using a modified version of ligation-mediated PCR (LM-PCR). Genomic DNA digestions used 60 U PvuII for 45 min at 37°C per 100 μ g DNA. The LM-PCR detection of cleaved DNA in each region of interest utilized the linker-specific primer LM-PCR1 (12) and the reverse primers used for ChIP of miRNA upstream regions listed in Table S1 in the supplemental material. Results were normalized to the cyclic threshold (C_T) values from 10% of undigested genomic DNA input amplified using oligonucleotides flanking the PvuII sites, which are the same oligonucleotides as those used for ChIP.

Tissue isolation and nucleus preparation from mouse skeletal muscle satellite cells and myofibers. The preparation of satellite cells and myofibers from the upper hind limb of 4-week-old C57/BL6 mice and the subsequent isolation of RNA and nuclei were performed as described previously (9, 35).

Mouse primary myoblast isolation and analysis. Primary myoblasts were isolated from the hind limb of postnatal day 3 mice as described previously (40). Primary myoblast cells from Brg1 conditional (42) or wild-type mice were infected at 50% confluence with the Adeno-cre virus (Ad5CMVCre) purchased from Gene Transfer Vector Core (University of Iowa) at a range of concentrations from 0.1 to 2 μ l (1 μ l = 2,250 PFU/cell). Alternatively, Brg1 myoblasts were infected with adenovirus expressing LacZ (Ad5CMVntLacZ; Gene Transfer Vector Core, University of Iowa) at concentrations of 0, 5, and 10 μ l (5 μ l = 3,750 PFU/cell). Upon reaching confluence, differentiation was initiated by the addition of low-serum media. Analysis occurred 4 days after the onset of differentiation. Cells were immunostained with myosin heavy chain (MyHC) antibody (MF20; Developmental Studies Hybridoma Bank) for myogenic differentiation, and the images were captured using a Zeiss Axioplan microscope. RNA was isolated pre- and postdifferentiation and subjected to qPCR analysis for the expression of miRNAs. Primers are listed in Table S1 in the supplemental material. DNA was extracted for testing for Brg1 excision by PCR using oligonucleotides TH185 and TG57 (42). The signal obtained for each sample was normalized to signal obtained by amplifying sequences upstream of the insertion site using primers defined in Table S1 in the supplemental material.

Zebrafish analysis. Morpholinos against Brg1 (5' CATGGTGGGTCAGG AGTGGACATC 3'), which is the same as the Brg1-MO2 used by others (17), and a control (5' CCTCTTACCTCAGTTACAAATTTATA3') were purchased from Gene Tools and dissolved in water at 100 mM each. Two hundred to 700 μ M of Brg1-MO or 700 μ M of control MO was injected into each embryo at the one- to two-cell stage and collected at 28 h postfertilization (hpf). RNA was isolated from control MO-injected embryos and from Brg1-MO-injected embryos showing a phenotype by TRIzol and subjected to RT-PCR to quantify the amount of specific miRNA transcripts using the oligonucleotides listed in Table S1 in the supplemental material. For immunostaining, Brg1-MO-injected embryos exhibiting a phenotype and control MO-injected embryos at 28 hpf were

fixed in 4% paraformaldehyde for 2 h. Embryos were incubated with primary antibody against sarcomeric α -actin (Sigma) or myosin heavy chain (MF20; Developmental Studies Hybridoma Bank) at 1:50 and 1:100 dilutions, respectively. MyHC-stained whole embryos were mounted in agarose and imaged with a Zeiss Axioplan microscope. For sarcomeric α -actin-stained embryos, tail muscle was dissected and mounted in agarose for imaging with a Leica SP confocal microscope.

RESULTS

Knockdown of the SWI/SNF ATPase Brg1 in zebrafish causes skeletal muscle defects and recapitulates the phenotype of nonfunctional Dicer mutant animals. As part of ongoing efforts to examine the function of Brg1 in development and differentiation, we injected Brg1-specific morpholino oligonucleotides (MO) or scrambled control MO into one- or two-cell-stage zebrafish embryos. Nearly all of the Brg1-MO-injected embryos showed visually apparent eye formation defects, which is consistent with the published phenotype (14, 17, 29). Approximately 40% of Brg1-MO-injected embryos showed defects in outgrowth and somite structure by 28 hpf (Fig. 1A to D). The fraction of affected embryos was constant for a range of 200 to 400 μ M MO per injection; above 400 μ M per injection, nearly all Brg1-MO-injected embryos failed early in development. Even at 700 μ M per injection, >90% of embryos receiving control MO developed normally. Most of the remainder failed early in development. None of the control MO-injected animals recapitulated the phenotype observed upon Brg1 MO injection. Injected embryos showing tail defects were stained for myosin heavy chain (MyHC). Control MO-injected animals showed characteristic MyHC staining, whereas the Brg1-MO-injected animals showed greatly reduced levels of staining (Fig. 1E and F). This is consistent with prior work demonstrating a direct requirement for Brg1-based SWI/SNF chromatin remodeling function at many myogenic gene regulatory sequences (12, 34).

To further analyze muscle structure in these animals, we immunostained samples with antibody against α -actin. We observed the significant disorganization of sarcomeric actin in Brg1-MO-injected embryos compared to the organization of control MO-injected embryos (Fig. 1G and H), with nearly a complete loss of the normal striations that occur in skeletal muscle tissue. The pattern of α -actin staining was strikingly similar to that observed when zebrafish with mutations in the microRNA processing enzyme, Dicer, or zebrafish injected with MO against the myogenic miRNAs, miR-1 and miR-133, were analyzed (31). As with MyHC, we observed a reduction in the expression of α -actin in zebrafish injected with Brg1-MO (Fig. 2A). These data suggest that Brg1 also contributes to sarcomeric actin organization during early zebrafish development. miR-1 and miR-133a are expressed in a skeletal and cardiac muscle-specific manner (6, 25, 48) and were shown to be required for myoblast proliferation and differentiation as well as for the maintenance of the differentiated state (6). We observed a significant reduction in the expression of miR-1-1, miR-1-2, miR-133a-1, and miR-133a-2 transcripts in the Brg1-MO-injected embryos, showing altered tail structures compared to that of control MO-injected embryos (Fig. 2B), thereby identifying myogenic miRNAs as targets for Brg1-based chromatin remodeling enzymes. Control experiments demonstrated that the expression of the Dicer enzyme and the

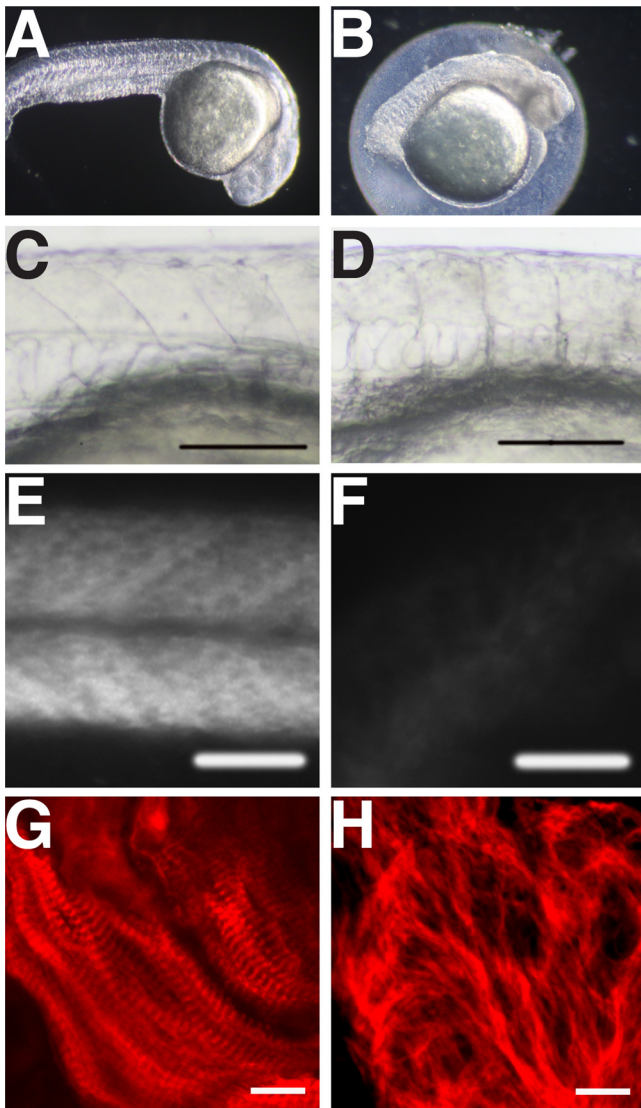


FIG. 1. Brg1-MO injection alters tail skeletal muscle organization. Zebrafish embryos were injected with control MO (A, C, E, and G) or Brg1-MO (B, D, F, and H), and animals were imaged at 28 hpf. (A and B) Normal and stunted tail development. (C) Normal somite structure was observed following control MO injection. (D) Altered somite structure was observed following Brg1-MO injection. Scale bar, 100 μ m. (E and F) MyHC staining in the tail following control or Brg1-MO injection. Scale bar, 50 μ m. (G and H) Tail skeletal muscle was immunostained for α -actin following control or Brg1-MO injection. Note the loss of I band staining in the sarcomeres in the Brg1-MO-injected tissue. Scale bar, 10 μ m.

widely expressed miRNA, miR-29a, were unaffected by Brg1-MO injection (Fig. 2A). Thus, Brg1 plays a previously unidentified role in myogenesis and skeletal muscle structural organization by promoting the expression of myogenic miRNAs. In addition, the results indicate that miRNA gene expression can be regulated by ATP-dependent chromatin remodeling enzymes.

Myogenesis and myogenic miRNA expression are compromised in Brg1 null mouse myoblasts. Primary myoblasts were isolated and cultured from the hind limb muscle of conditional

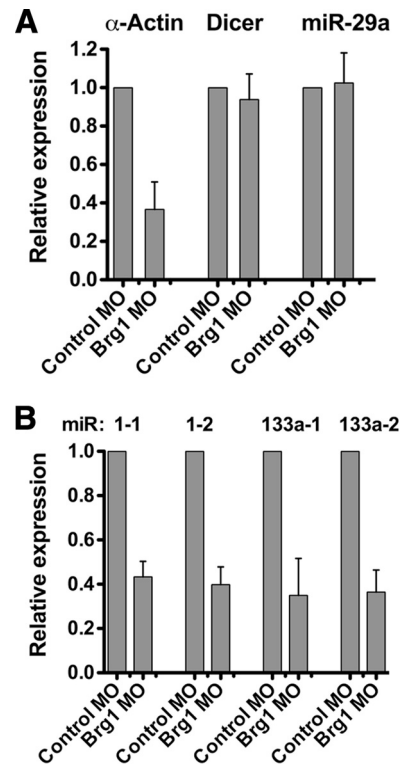


FIG. 2. Brg1-MO-injected zebrafish with altered tail development have compromised α -actin and skeletal muscle microRNA expression. (A) qPCR analyses of α -actin, Dicer, and miRNA-29a primary transcript levels in control- or Brg1-MO-injected animals. (B) qPCR to detect the indicated miRNA primary transcript levels in control MO-injected embryos or in Brg1-MO-injected animals that exhibited a tail phenotype. The expression of each gene in the control MO-injected animals was normalized to 1; results are the averages from three independent experiments performed on different days \pm standard deviations. For each independent experiment, eight or nine embryos were pooled for RNA preparation.

Brg1 (42) and wild-type mice (40). Adenovirus encoding Cre-recombinase (Ad-Cre)-infected and control cells were differentiated into myotubes *ex vivo* by replacing the growth media with low-serum differentiation media for 4 days. Cre-infected conditional Brg1 myoblasts did not differentiate, whereas the control conditional Brg1 formed myotubes (Fig. 3A). As expected, wild-type cells with or without Ad-Cre infection also differentiated (Fig. 3B, top and middle). As an additional control, Brg1 conditional myoblasts were infected with an adenovirus encoding lacZ (Ad-lacZ); these cells differentiated normally (Fig. 3B, bottom). To confirm that the Brg1 conditional allele was excised by Ad-Cre, increasing concentrations of virus were used to infect Brg1 conditional myoblasts, and a PCR-based analysis was performed to monitor gene excision (Fig. 4A). Western blots from Ad-Cre-infected samples demonstrated that gene excision resulted in a reduction of Brg1 protein levels (Fig. 4B).

Having demonstrated that Brg1 is required for myoblast differentiation *ex vivo*, we examined whether microRNA expression was compromised in the Brg1-deficient cells. The analysis of myogenic miRNA transcripts in Brg1-ablated cells showed significant reduction in the levels of miR-1-1, miR-1-2,

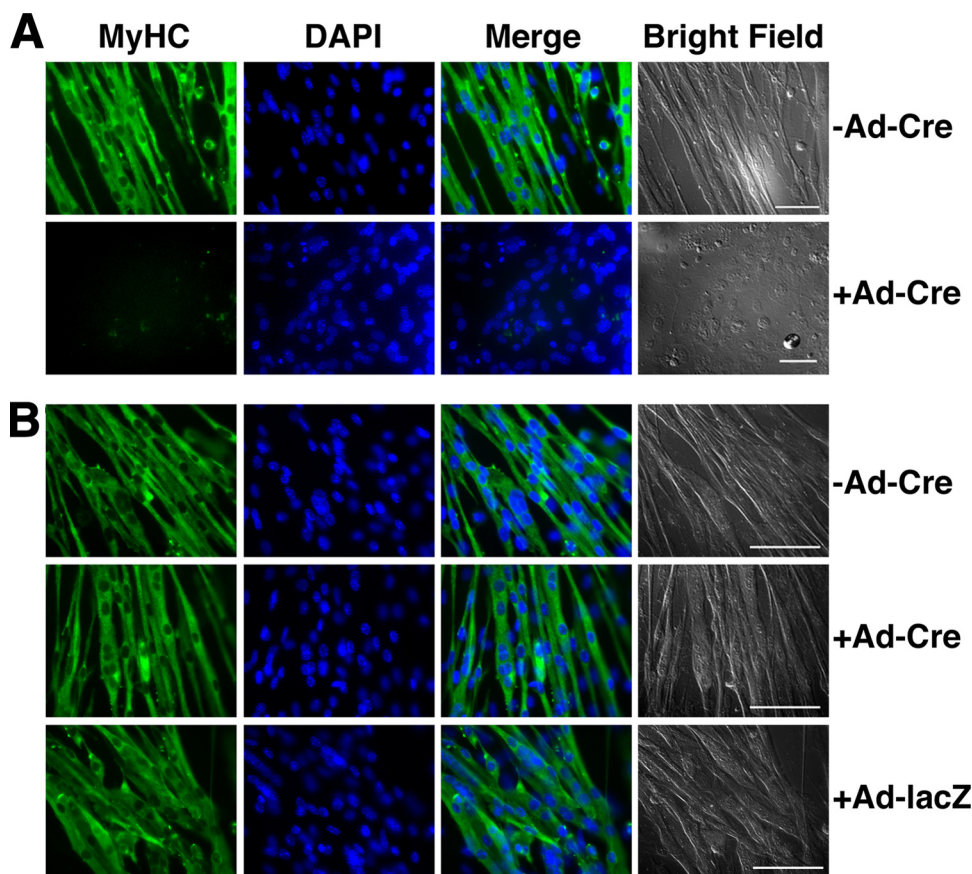


FIG. 3. Brg1 conditional myoblasts treated with Ad-Cre do not differentiate. (A) Myoblasts isolated from newborn Brg1 conditional mice were cultured, mock infected, or infected with 0.5 μ l (1,125 PFU/cell) Ad-Cre, and then they were induced to differentiate by the addition of low-serum media. Four days postdifferentiation, cells were stained for MyHC and with 4',6'-diamidino-2-phenylindole (DAPI) and compared to bright-field images. (B) The same experiment as that described for panel A was repeated with myoblasts isolated from wild-type mice (top and middle) or with Brg1 conditional myoblasts infected with 5 μ l (3,750 PFU/cell) of Ad-lacZ (bottom). Scale bar, 50 μ m.

miR-133a-1, and miR-133a-2 transcripts upon Ad-Cre infection but no change in the transcript levels of the widely expressed miR-29a (Fig. 4C). The induction of primary transcripts of miR-1 and miR-133a upon differentiation was not affected when wild-type myoblasts were infected with Ad-Cre (Fig. 4D), nor were microRNA primary transcript levels affected when Brg1 conditional myoblasts were infected with Ad-lacZ (Fig. 4E). A role for Brg1 in the induction of myogenic miRNAs therefore is conserved between fish and mammals. We note that the ablation of Dicer in mice also resulted in altered myofiber organization, decreased muscle mass, and decreased miRNA levels (33), providing additional support for the idea that these factors are functionally linked in vertebrate myogenesis.

Brg1 binds to and remodels miRNA regulatory regions. To begin to explore the mechanism by which Brg1 affects miRNA induction, we isolated tissue from mouse hind limb muscle and separated mature myofibers from the satellite cells, which are responsible for muscle generation and regeneration in the postnatal organism. The expression of miR-1-1, miR-1-2, miR-133a-1, and miR-133a-2 primary transcripts as well as the mature forms of miR-1 and miR-133a were significantly induced in satellite cells and myofibers compared to levels in

liver tissue (Fig. 5). miR-1 and miR-133a are transcribed by two bicistronic miRNA clusters encoding miR-1-1/miR-133a-2 and miR-1-2/miR-133a-1 (6, 30, 48). Several E boxes, which are binding sites for MyoD and related myogenic determination factors, were identified upstream of miR-1-1, miR-1-2, miR-133a-1, and miR-133a-2 stem-loop sequence using TRANSFAC-Professional software. The locations of consensus E boxes in regions likely to regulate miRNA expression are shown in Fig. 6A; prior work has indicated that myogenic regulatory factors can bind to many of these E boxes in differentiating C2C12 myoblasts (36).

Chromatin immunoprecipitation (ChIP) demonstrated that both MyoD (Fig. 6B) and Brg1 (Fig. 6C) interacted with miR-1-1 upstream regions containing all but the two most distal E boxes specifically in the muscle tissues, not in liver tissue. MyoD and Brg1 also interacted with the upstream regions of miR-1-2 and miR-133a-1 and with the proximal, but not distal, region of miR-133a-2 in a muscle tissue-specific manner (Fig. 7A and B). IgG ChIPs and the amplification of the IgH enhancer, which contains a consensus E box, were performed as controls for these and all subsequent ChIP experiments; in no case did any control signal exceed background (Fig. 7 and data not shown).

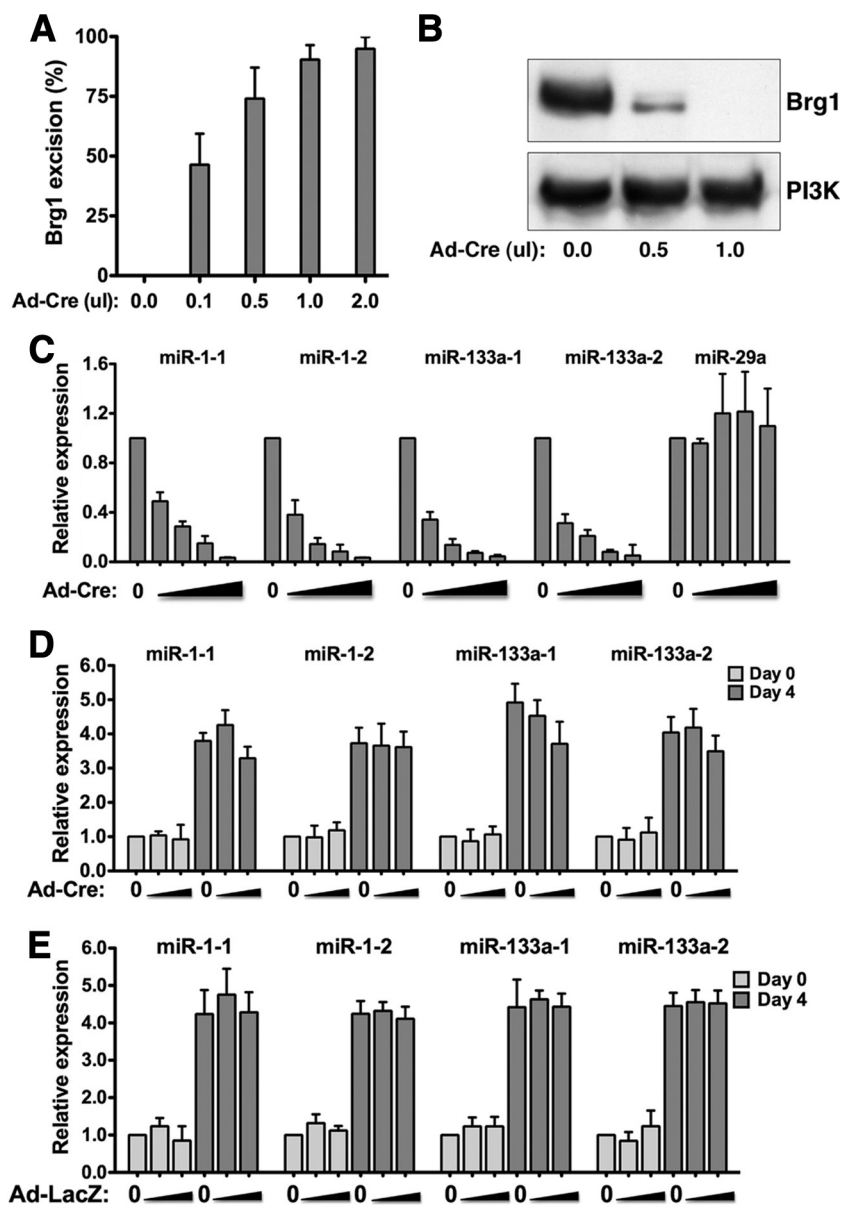


FIG. 4. Brg1 conditional myoblasts treated with Ad-Cre are impaired in myogenic microRNA expression. (A) Excision of the Brg1 conditional allele as a function of an increasing dose of Ad-Cre (0, 0.1, 0.5, 1, and 2 μ l; 1 μ l = 2,250 PFU/cell). (B) Western blot demonstrating Brg1 protein levels in Brg1 conditional myoblasts treated with 0, 0.5, or 1 μ l of Ad-Cre. PI3 kinase (PI3K) levels were monitored as a control. (C) Relative miRNA primary transcript levels present in Brg1 conditional myoblasts infected with increasing amounts of Ad-Cre (0, 0.1, 0.5, 1, and 2 μ l) and assayed at day 4 postdifferentiation. (D) Relative expression of miR-1 and miR-133a primary transcripts in wild-type myoblasts infected with 0, 1, or 2 μ l of Ad-Cre and assayed at day 0 or 4 postdifferentiation. (E) Relative expression of miR-1 and miR-133a primary transcripts from Brg1 conditional myoblasts infected with Ad-LacZ (0, 5, or 10 μ l; 5 μ l = 3,750 PFU/cell) and assayed at day 0 or 4 postdifferentiation. MicroRNA primary transcript levels presented in panels C to E were quantified in three independent experiments and are presented as averages \pm standard deviations. The expression in the absence of Ad-Cre or Ad-lacZ was normalized to 1.

We subsequently used a restriction enzyme accessibility assay (REAA) to demonstrate that the accessibility of chromatin at the upstream regulatory regions of miR-1-1 that were bound by MyoD and Brg1 was increased specifically in muscle tissue compared to levels in liver (Fig. 6D). We also detected increased restriction enzyme accessibility at miR-1-2, miR-133a-1, and miR-133a-2 upstream regulatory regions, where both MyoD and Brg1 were bound in a muscle tissue-specific manner (Fig. 7C). No change in restriction enzyme accessibil-

ity was observed at the E box present in the coding region of the constitutively expressed Eef1 α 1 gene that was used as a control (Fig. 7C). These data demonstrate that in primary muscle tissue, Brg1 localizes to the upstream regions of myogenic miRNA stem-loop sequences and Brg1 binding correlates with the binding of the MyoD regulator and open chromatin structure, suggesting that Brg1 and MyoD are promoting chromatin remodeling and the expression of myogenic miRNAs.

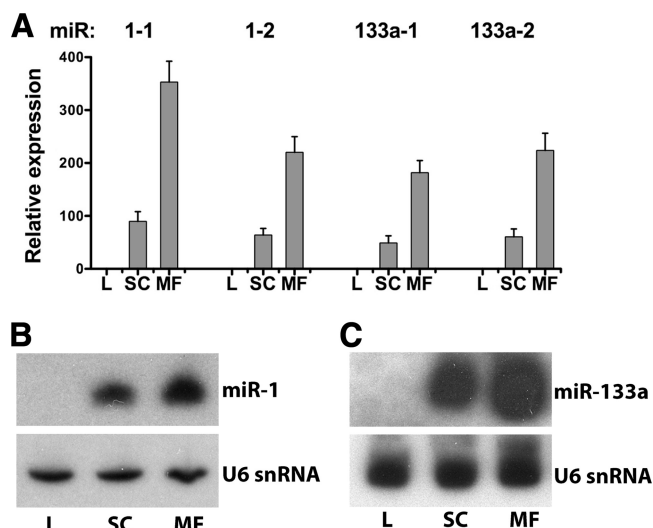


FIG. 5. Myogenic microRNAs are highly induced in primary skeletal muscle tissues. (A) Relative expression levels of miRNA primary transcripts miR-1-1, miR-1-2, miR-133a-1, and miR-133a-2 in liver (L), satellite cells (SC), or myofibers (MF). Transcript levels in liver tissue were normalized to 1. (B and C) Northern blots of mature miR-1 and miR-133a levels in these tissues. U6 snRNA was measured as a loading control.

Myogenic miRNA expression requires functional Brg1. B22 fibroblast cells inducibly express ATPase deficient, dominant-negative Brg1 (DN Brg1) upon the removal of tetracycline from the culture medium (10). To further examine how Brg1 modulates the expression of the muscle-specific microRNAs and to directly assess the requirement for Brg1 enzymatic activity, these cells were grown in the presence or absence of tetracycline and then directed along the skeletal muscle lineage via infection with retrovirus encoding MyoD. The analysis of cells at the onset of differentiation and postdifferentiation confirmed earlier work demonstrating the inducible expression of FLAG-tagged DN Brg1 and the inhibition of representative early and late myogenic genes (11 and data not shown). Northern blot analyses for the detection of the mature form of miR-1 and miR-133a showed the induction of the expression of both miRNAs upon myogenic differentiation and a significant reduction in miRNA levels in the cells expressing DN Brg1 (Fig. 8A and B). However, the expression of a widely expressed miRNA, miR-29a, was not affected by differentiation or by the expression of DN Brg1 (Fig. 8C). Analyses of miR-1 and miR-133a primary transcripts by qPCR confirmed these results (Fig. 9A). These data indicate that functional Brg1 is required for the induction of miR-1 and miR-133a upon skeletal muscle differentiation in these cells.

Subsequent ChIP studies indicated that MyoD and Brg1 were present at the same regions upstream of myogenic miR-1-1 stem-loop sequences in the differentiated B22 cells, as had been observed in primary tissue (Fig. 9B and C). The same results were observed upstream of miR-1-2, miR-133a-1, and miR-133a-2 stem-loop sequences (Fig. 10A and B). MyoD binding was unaffected by the presence of DN Brg1, indicating that MyoD binding at these loci is independent of chromatin remodeling by Brg1-based SWI/SNF complexes and suggesting that the targeting of Brg1 likely is mediated via physical inter-

actions with MyoD-bound chromatin, as has been demonstrated previously at some myogenic loci (12, 39). We also noted that no Brg1 binding was observed when DN Brg1 was expressed. We previously showed that the induction of DN Brg1 causes the level of endogenous Brg1 protein to be severely diminished and proposed that the autoregulation of Brg1 levels occurs in cells (12). This explains why no endogenous Brg1 was observed in the ChIP experiments. The reason DN Brg1 is not targeted to any of the miRNA loci is not understood; however, we previously showed that DN Brg1 also does not ChIP to regulatory sequences controlling Brg1-dependent genes expressed at late times of the differentiation process (34). Regardless, the lack of functional Brg1 present at the miRNA upstream sequences predicts that chromatin remodeling does not occur at these loci; the REAA of the miR-1-1 upstream region in differentiated cells showed increased chromatin accessibility at each region occupied by Brg1 and no change in chromatin accessibility when DN Brg1 was expressed (Fig. 9D). The same results were observed at sequences upstream of the other myogenic miRNA loci (Fig. 10C). The results support the idea that Brg1 is targeted to regulatory regions upstream of myogenic miRNA sequences by MyoD and locally remodels chromatin structure, which leads to the induction of myogenic miRNA transcripts.

DISCUSSION

The remarkable similarity of phenotypes between zebrafish having reduced levels of Brg1, mutated Dicer enzyme, or reduced levels of myogenic miRNAs suggested that Brg1, Dicer, and myogenic miRNAs function in the same pathway *in vivo* to regulate proper skeletal muscle development and structure. In zebrafish muscle, miR-1 and miR-133 account for more than 50% of the total miRNA-mediated gene regulation (31). These two microRNAs target mRNAs encoding proteins associated with actin function, and the knockdown of these miRNAs or of the Dicer enzyme results in altered actin organization in muscle sarcomeres (31). A notable feature of the altered actin organization in zebrafish lacking miR-1 and miR-133 or in zebrafish lacking Dicer is the near absence of striation and an inability to distinguish I bands. Strikingly, when the actin organization in Brg1-MO-injected fish with stunted tails was examined, a similarly altered organization, including the lack of distinct bands in the sarcomeres, was observed. These data indicate that in an *in vivo* setting, deficiency in Brg1 equates with the deficiency in Dicer or deficiency in myogenic microRNAs. The simplest explanation is that Brg1, Dicer, and myogenic microRNAs function in the same pathway to regulate actin organization in skeletal muscle tissue. Although there are multiple means by which these proteins might functionally relate to each other, a likely explanation given the known properties of these molecules is that the Brg1 chromatin remodeling enzyme is required for the expression of Dicer or the microRNAs. Direct analysis revealed that Dicer levels were unaffected in the Brg1-MO-injected zebrafish, whereas myogenic microRNA expression was compromised (Fig. 1). Thus, we identified myogenic microRNAs as target genes for Brg1 in the zebrafish.

We note that numerous zebrafish genes involved in retinal formation and function have been identified as Brg1 targets

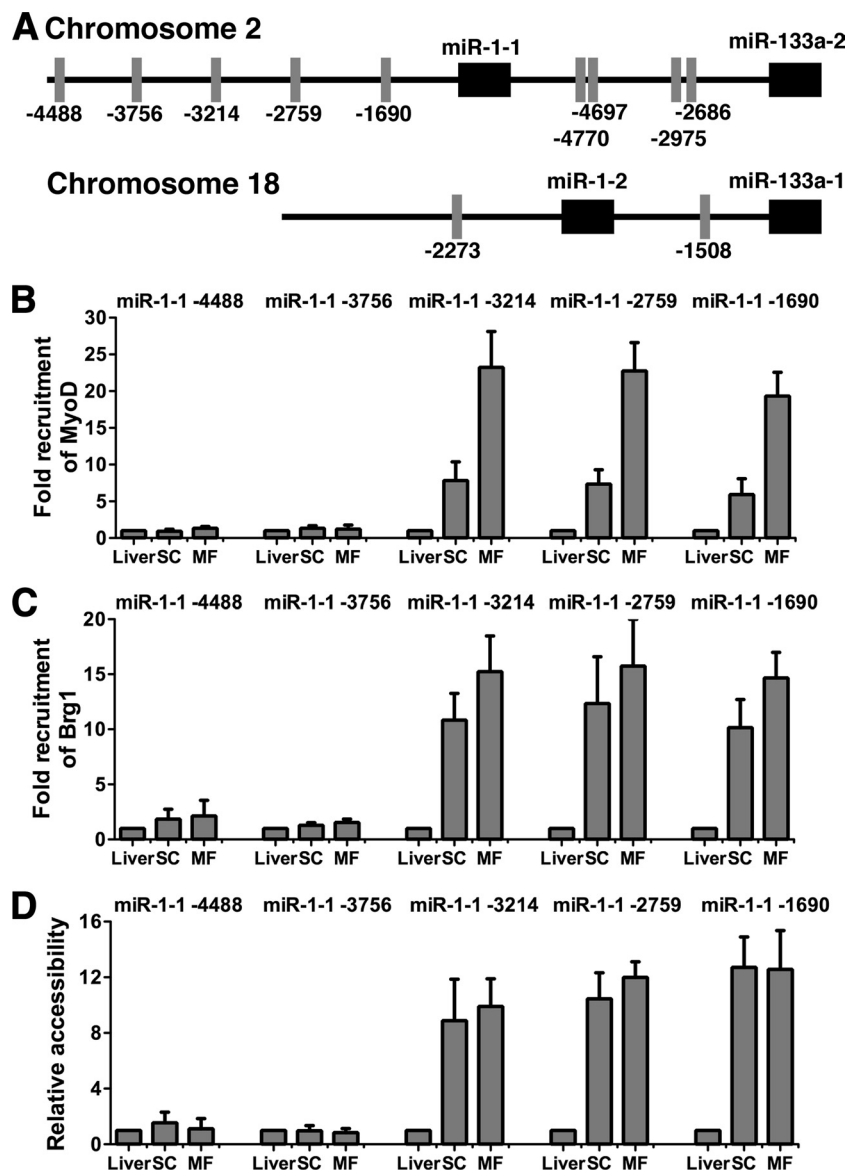


FIG. 6. Brg1 binding correlates with chromatin remodeling near all but the most distal of the E boxes upstream of the miR-1-1 stem-loop sequence in primary tissue. (A) Schematic maps of the miR-1 and miR-133 loci. The positions of consensus E boxes upstream of each miRNA stem-loop sequence are indicated. (B and C) ChIP experiments demonstrating the binding of MyoD (B) and Brg1 (C) near all but the most distal E boxes from the miR-1-1 stem-loop sequence in satellite cells (SC) and myofibers (MF) but not liver tissue. Levels of MyoD or Brg1 binding at each sequence in the liver sample were normalized to 1. (D) REAA from the tissue samples used for panels B and C indicating increased nuclease accessibility at PvuII sites (which exactly correspond to E boxes) at all but the most distal E boxes from the miR-1-1 stem-loop sequence. Enzyme cleavage at each sequence in the liver sample was normalized to 1. Data in panels B to D are the averages from three independent tissue isolations \pm standard deviations.

(27), and that in mammalian systems, Brg1 and SWI/SNF enzymes have been identified as regulators of genes involved in nearly every tissue differentiation process examined (13), including skeletal muscle (11, 12, 39). Thus, this work extends the role of Brg1 beyond the regulation of mRNAs to include the regulation of microRNA expression. Because Brg1 is not solely required for miRNA production, we did not attempt to rescue the Brg1 deficiency by the introduction of miR-1 and miR-133. In addition to the likelihood that there are other myogenic microRNAs that are deficient due to the reduction in Brg1 levels, myogenic mRNA expression also would be

compromised, rendering the attempt at rescuing the phenotype of Brg1 unproductive. We note, however, that other workers have rescued retinal and neurogenesis defects due to Brg1 morpholino injection via the introduction of either a Brg1 cDNA or a genomic P1-derived artificial chromosome that includes the Brg1 locus (17) or via the introduction of Brg1 mRNA (14). The morpholino used in our experiments was the same one used by Gregg et al. (17), suggesting that off-target effects are not the cause of the observed phenotypes.

miR-1 and miR-133, in addition to being expressed in skeletal muscle, are expressed in cardiac muscle (6, 25, 48). Brg1 and

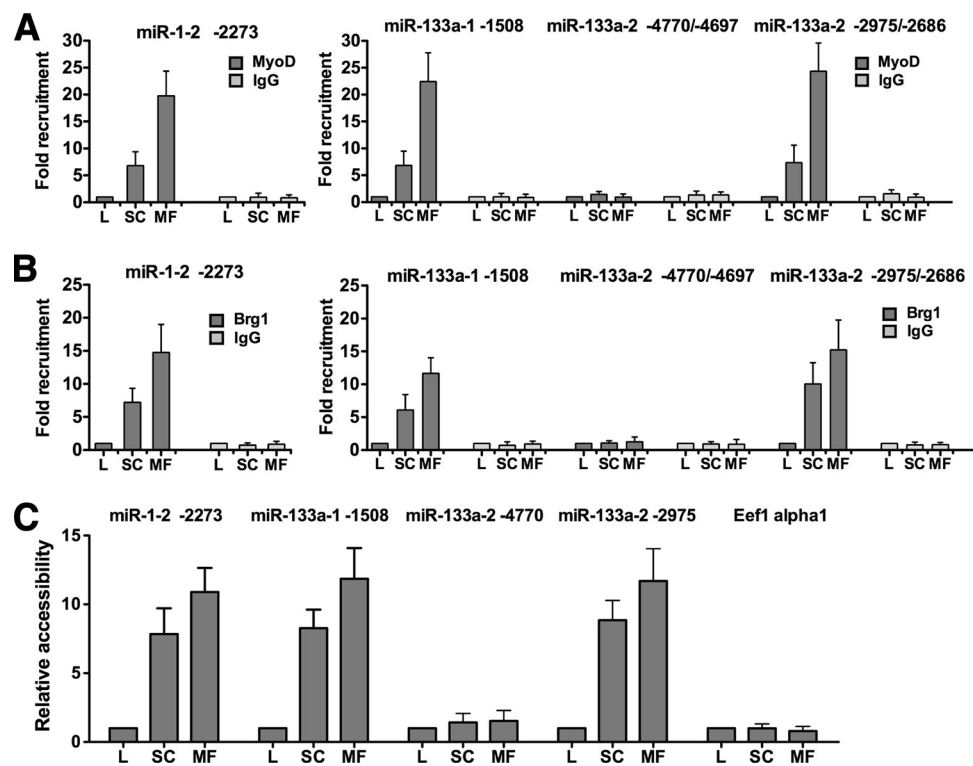


FIG. 7. MyoD binding, Brg1 binding, and chromatin accessibility correlate at E box regions of miR-1-2, miR-133a-1, and miR-133a-2 in primary tissue. MyoD (A) and Brg1 (B) recruitment at the indicated E box-containing sequences upstream of the miR-1-2, miR-133a-1, and miR-133a-2 coding sequences in liver (L), satellite cells (SC), or myofibers (MF). Levels of MyoD or Brg1 binding at each sequence in the liver sample were normalized to 1. Background binding observed with IgG pulldown in each tissue is shown as a control. (C) REAA at the indicated E boxes upstream of the miR-1-2, miR-133a-1, and miR-133a-2 coding sequences in liver (L), satellite cells (SC), or myofibers (MF). Enzyme cleavage at each sequence in the liver sample was normalized to 1. Accessibility at the E box in the *Eef1* α 1 coding region was examined as a control. Data are the averages from three independent tissue isolations \pm standard deviations.

two other subunits of the mammalian SWI/SNF enzyme complex, Baf180 and Baf60, have been shown to contribute to cardiac development and function in mouse models (20, 28, 41, 45). This raises the question of whether the Brg1-MO-injected fish also showed heart defects. Although we did not perform any heart analysis in these animals, we observed that beating hearts were present in all animals showing skeletal muscle defects (data not shown). However, we did note pericardial swelling in animals that survived to 48 hpf and later time points (data not shown). Pericardial swelling was a reported characteristic of the *yng* mutant fish line (29), which was later shown to be mutated in the Brg1 gene (17). Thus, the nonskeletal muscle phenotypes observed in our Brg1-MO-injected zebrafish are consistent with previous reports.

We performed subsequent studies to address the requirement for and the mechanism controlling the function of Brg1 in myogenic microRNA regulation via the direct analysis of skeletal muscle tissue and by the manipulation of Brg1 levels in primary cells cultured *ex vivo* and in a tissue culture model for myogenesis. Although we cannot definitively state that Brg1 is directly acting at microRNA regulatory sequences in zebrafish skeletal muscle tissue, the data obtained from these multiple experimental systems all are consistent with the conclusion that Brg1 promotes chromatin remodeling and gene expression at myogenic miRNA regulatory sequences. Our analysis of MyoD binding to regions upstream of individual myogenic microRNA

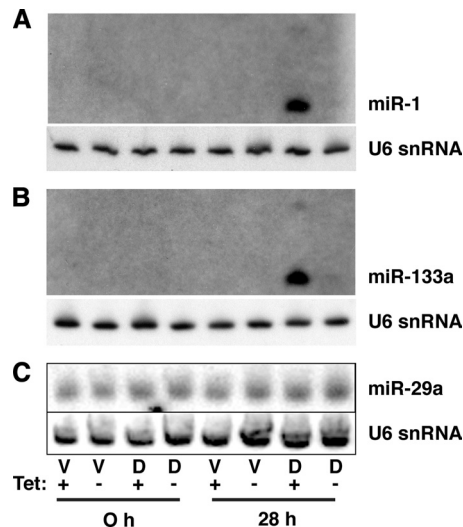


FIG. 8. Expression of miRNAs miR-1 and miR-133a is compromised in differentiated cells expressing DN Brg1. B22 cells express dominant-negative Brg1 in the absence but not the presence of tetracycline (Tet). B22 cells cultured in the presence or absence of Tet were infected with the pBABE retroviral vector (V) or pBABE-MyoD (D), and samples were collected at the onset of differentiation, designated time zero, and subsequently differentiated by the addition of a low-serum medium for 28 h. (A and B) Northern blots for miR-1 and miR-133a miRNAs in differentiated B22 cells. (C) Expression of the widely expressed miR-29a miRNA was monitored by Northern blotting as a control. U6 snRNA levels were measured as a loading control for each blot.

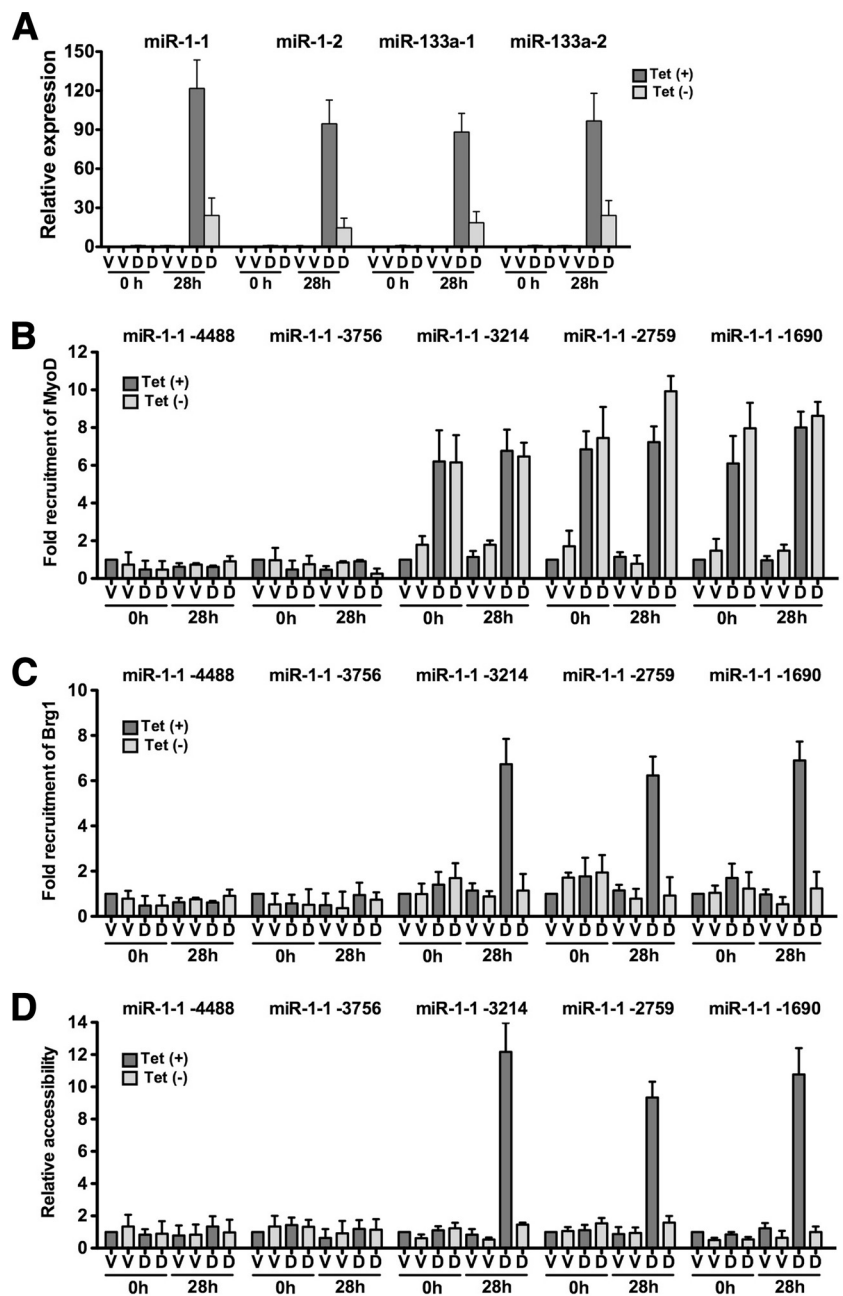


FIG. 9. Functional Brg1 is required for chromatin remodeling and gene expression from the miR-1-1 locus in cultured cells. (A) B22 cells cultured without tetracycline (Tet) express DN Brg1. Cells were infected with empty retroviral pBABE vector (V) or with a MyoD-encoding retrovirus (D) and were collected at the onset of differentiation, which was designated 0 h, or at 28 h postdifferentiation. Cells expressing DN Brg1 showed significantly reduced levels of miR-1-1, miR-1-2, miR-133a-1, and miR-133a-2 primary transcripts compared to those of cells grown with Tet. (B and C) ChIP experiments examining the presence of MyoD or Brg1 near E boxes upstream of the miR-1-1 gene in the samples described for panel A. (D) REAA experiments examining chromatin accessibility in these samples at the indicated E boxes. All experiments are the averages of three independent experiments \pm standard deviations, and expression, binding, or enzyme cleavage at each sequence in the vector-differentiated cells plus tetracycline at time zero was normalized to 1.

coding sequences in both primary tissue and tissue culture cells revealed MyoD interactions with at least one consensus E box located within 3 kb of each of the mature microRNA start sites. However, additional consensus E boxes further upstream did not show interactions with MyoD. Interestingly, the expression of dominant-negative Brg1 did not inhibit MyoD binding to these sites, indicating that MyoD is able to access these se-

quences in the absence of functional chromatin remodeling by Brg1-based SWI/SNF complexes. This is similar to observations made regarding MyoD binding to myogenic genes encoding mRNAs that are expressed at later times of differentiation (34) but distinct from events at the myogenin promoter, where the homeodomain factor Pbx-1 initiates the cascade of transcription factor binding and chromatin remodeling (3, 12).

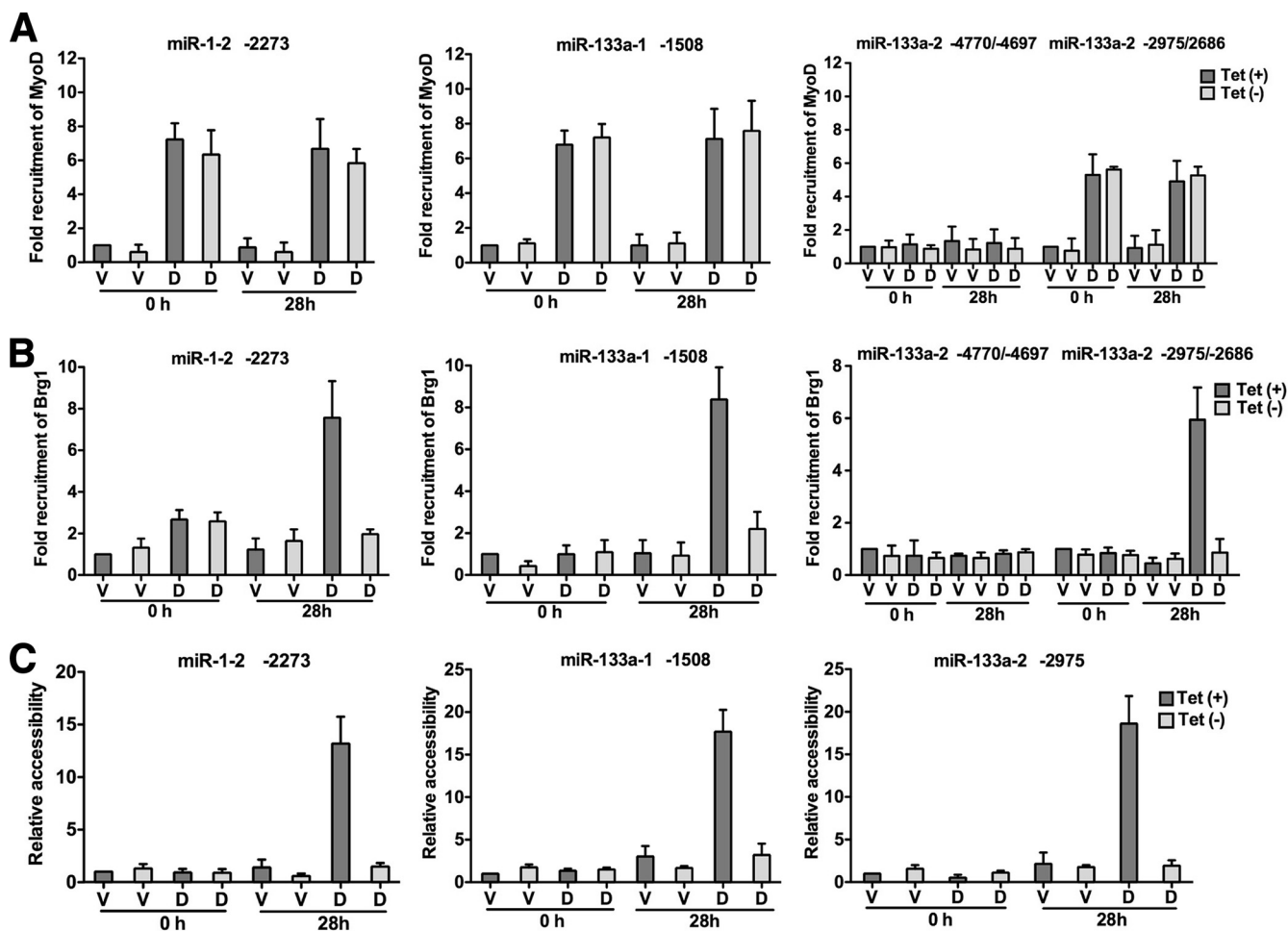


FIG. 10. Expression of DN Brg1 affects Brg1 binding and chromatin accessibility at upstream E boxes of miR-1-2, miR-133a-1, and miR-133a-2 stem-loop sequences. MyoD (A) and Brg1 (B) ChIPs at E box-containing sequences upstream of the miR-1-2, miR-133a-1, and miR-133a-2 gene sequences in B22 cells treated as described for Fig. 9. (C) REAA at the indicated E boxes upstream of the miR-1-2, miR-133a-1, and miR-133a-2 genes. Binding or enzyme cleavage at each sequence in the vector-differentiated cells plus tetracycline at time zero was normalized to 1. Data represent the averages from three independent experiments \pm standard deviations.

Whether MyoD possesses an intrinsic ability to interact with its binding sites upstream of myogenic microRNAs or whether different chromatin modifying or remodeling events are required remains unknown.

ChIP experiments showed that the Brg1 ATPase of SWI/SNF enzymes was present at precisely the same sequence regions upstream of myogenic microRNAs as those that were bound by MyoD. Again, there was exact agreement between Brg1 binding in muscle tissue and binding in tissue culture cells. The data suggest that the Brg1 remodeling enzyme is targeted to the sequences upstream of the myogenic microRNAs by MyoD. This regulatory event is consistent with prior data showing that MyoD coimmunoprecipitates with Brg1 from cell extracts and targets Brg1 to some myogenic protein coding genes (12, 39). The functionality of Brg1 was demonstrated by the exact correlation, again both in muscle tissue and in tissue culture cells, between the presence of MyoD and wild-type Brg1 and increased nuclease accessibility at sites of MyoD and Brg1 binding, while sites not bound by MyoD and Brg1 showed no change in chromatin accessibility.

Collectively, these data indicate that Brg1 is required for skeletal muscle organization and that a previously unappreciated function of the Brg1-based SWI/SNF chromatin remodeling enzyme during myogenesis is to promote the expression of myogenic miRNAs that are important contributors to vertebrate myogenic development and function. This work establishes the concept that the tissue-specific induction of microRNA expression, like mRNA expression, is regulated by Brg1-based ATP-dependent chromatin remodeling enzymes.

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